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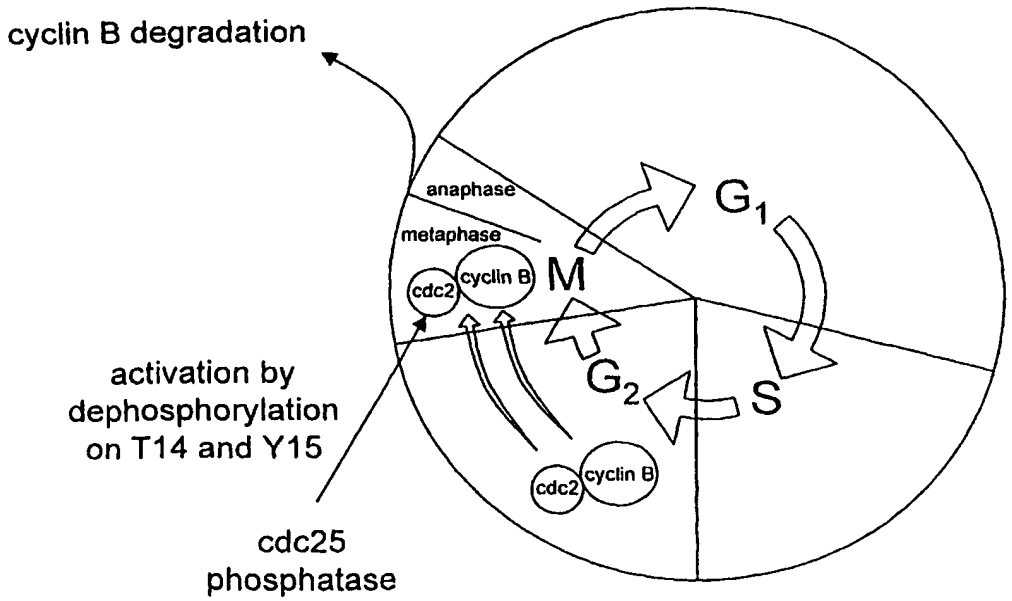

(57) Abstract

The present invention relates to the use of indigoid bisindole derivatives for the manufacture of a medicament for inhibiting cyclin dependent kinases, particularly CDK 1, CDK 2, CDK 4 and CDK 5, more particularly ATP:Proteinphosphotransferase $p34^{cdc2}$ (CDK1).

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"Use of indigoid bisindole derivatives for the manufacture of a medicament to inhibit cyclin dependent kinases"

Description

The present invention relates to the use of indigoid bisindole derivatives for the manufacture of a medicament for inhibiting cyclin dependent kinases, particularly CDK 1, CDK2, CDK 4 and CDK 5, more particularly ATP:Proteinphosphotransferase $p34^{cdc2}$ (CDK1).

Indigoid bisindoles comprise a spectrum of natural dye stuffs. Many of these can be obtained from plants. Accordingly, indirubine, indigo and isoindigo are natural products which can be obtained from different plants: namely, Baphicacanthus cusia (Acanthaceae), Indigofera suffruticosa (Fabaceae), Isatis indigotica (Brassicaceae) and others. Indican, a glycoside which is found in plants, gives glucose and 3-hydroxyindole due to acidic or enzymatic hydrolysis. 3-Hydroxyindole is converted by air-oxidation into indigo and its isomers. Indigo naturalis (Chinese: quingdai) is the natural blue dye obtained from plant material, e.g. Isatis indigotica (Brassicaceae). Indirubine, an isomer of indigo, can be found in Indigo naturalis in an amount of up to 60% (Falbe J. & Regitz M., Römpp Chemie Lexikon (1992), 9. Aufl., Stuttgart, Georg Thieme Verlag). It occurs also in Isatis tinctoria in an amount of up to 5% which is indigenous to Central Europe (Gelius R., Z. Chem., 20, (1980), 340-341).

Indigo naturalis is reported to be used in traditional Chinese medicine as a hemostatic, antipyretic, anti-inflammatory and sedative agent in the treatment of bacterial and viral infections. Antileukemic effects of Indigo naturalis have also been reported, with indirubine being the effective principle (Ji X. et al., Acta Pharm. Sin., 16, (1981), 146-148; Gan W. J. et al., J. Hematol., 6, (1985), 611-613). Furthermore, derivatives of indirubine are known for a long time as dyes of low persistence.

Few, structurally quite different types of $p34^{cdc2}$ inhibitors have been described up to now. They are either of natural origin or derived from natural compounds and show varying degrees of inhibitory activities. Examples are Staurosporine, an alkaloid from Streptomyces sp, Butyrolactone-I from Aspergillus terreus var., Flavopiridol, a novel promising anti-tumour agent derived by partial synthesis from a parent structure found in the indian plant Dysoxylum binectariferum, 9-Hydroxyellipticin from the plants Ochrosia elliptica and Ochrosia acuminata, the purine derivatives olomoucine, roscovitine and isopentenyladenine and some peptides. The mechanism of these compounds is based on competitive inhibition of ATP binding (Meijer L., Trends in Cell Biology, 6, (1996), 393-397).

For determination of the IC 50-values: $p34^{cdc2}$/cyclin B was purified from M phase starfish (Marthasterias glacialis) oocytes by affinity chromatogaphy on $p9^{CKShs1}$-Sepharose beads, from which it was eluted by free $p9^{CKShs1}$ as described (Meijer et al., Eur. J. Biochem., 243, (1997), 527-536). It was assayed with 1 mg histone H1 (Sigma type III-S)/ml, in the presence of 15 μM [γ-^{32}P] ATP (3,000 Ci/mmol; 1mCi/ml) in a final volume of 30 μl. After 10 min. incubation at 30°C, 25 μl aliquots of supernatant were spotted onto 2.5 x 3 cm pieces of Whatman P81 phosphocellulose paper, and after 20 sec., the filters were washed five times (for at least 5 min. each time) in a solution of 10 ml phosphoric acid/liter of water. The wet filters were transferred into 6 ml plastic scintillation vials, 5 ml ACS (Amersham) scintillation fluid was added and the radioactivity measured in a Packard scintillation counter. The kinase activity was expressed in pmoles phosphate incorporated into histone H1/10 min incubation or in % of maximal activity.

The strongest inhibitors of $p34^{cdc2}$ are derivatives of staurosporine (IC_{50} = 0,003-0,03 μM). The selectivity of these inhibitors is, however, rather poor. They also show more or less potent inhibitory activity to quite a wide range of cellular kinases. Table 1 shows the specifity of known chemical inhibitors of cyclin-dependent kinases (IC_{50}-values given in μM) (cf. Meijer L., Trends in Cell Biology, 6, (1996), 393-397).

Tab. 1:

Enzyme	Staurosporine	UCN-01	Butyrolactone-I	Flavopiridol	Olomoucin	Roscovitine	9-Hydroxy-ellipticine
CDK1	0.003-0.009	0.031	0.60	0.40	7	0.65[a]	ca. 1
CDK2	0.007	0.030	1.50	0.40	7	0.70	ND
CDK4	< 10.000	0.032	no effect	0.40	>1000	>100	ND
MAPK	0.020	0.910	94	ND	30	30	ND
PKA	0.008	ND	260	145	>2000	>1000	ND
PKG	0.009	ND	ND	6	>2000	>1000	ND
PKC	0.005	0.007	160	ND	>1000	>100	ND
Tyrosine-kinase	0.006 (EGF-R) 0.006 (Src)	ND	>590 (EGF-R)	25 (EGF-R)	440 (EGF-R)	70 (I-R)	ND

CDK: cyclin-dependent kinase; EGF-R: epidermal growth factor receptor tyrosine kinase; I-R: insulin receptor-tyrosine kinase; MAPK: mitogene-activated protein kinase; ND: not determined; PKA: cAMP-dependent protein kinase; PKG: cGMP-dependent protein kinase.

[a]IC_{50}-value for racemic mixture; IC_{50} for (R)-roscovitin is 0.45 μM.

Cyclins and cyclin dependent kinases (CDK) have an essential role for driving the cell through the cell cycle (cell division cycle, cdc). During the cell division cycle, oscillations in concentrations and activities of cyclins are observed. This applies, e.g. to cyclins D and E in the so-called G1-phase of the cell cycle and to cyclinA (S- and M-Phase) and cyclinB (G2- and M-Phase).

The cyclin dependent kinases are activated by association with a member of the cyclin-family. Up to now, eight human CDKs have been described: CDK1 (= $p34^{cdc2}$), CDK2 to CDK8. CDK-proteins consist of a catalytic subunit and a regulatory subunit, the cyclins (Meijer et al., Eur. J. Biochem., 243, (1997), 527-536). Every step of the cell division cycle is regulated by specific CDK/cyclin complexes which ascertain a strict control. Important checkpoints are at the transition from G1 phase to S phase and from G2 phase to M phase (Pines J.,

Cancer Biology, 6, (1995), 63-72). The $p34^{cdc2}$/cyclinB complexes are important components at the G_2-M-checkpoint.

Fig.1 shows the points of action of the cyclin CDK complex cdc2/cyclinB in the cell divsion cycle (M= mitosis, cell division; G = gap; S = synthesis; interphase = G_1+S+G_2).

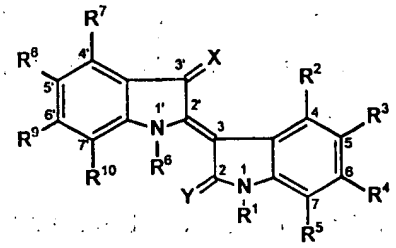
The cdc2/cyclinB complexes are the primary active protein kinases in mitosis. They accumulate in an inactive state in the cytoplasm in interphase cells, and are then rapidly activated by cdc25 phosphatase and translocated into the nucleus at the beginning of mitosis (Pines J., Hunter J., Cell Biol., 115(1), (1991),1-17). CyclinB is degraded at the metaphase-anaphase transition, inactivating cdc2, which is necessary for exit from mitosis (Glotzer et al., Nature, 349(6305), (1991),132-138; Murray A.W., Nature, 339(6222), (1989), 280-286; Surana U. et al., Cell, 65(1), (1991), 145-161). Multiple changes of CDK proteins and their regulators are associated with the development of human tumours (Cordon-Cardo C., Am. J. Pathol., 147(3), (1995), 545-560).

Thus, the technical problem underlying the present invention is to provide new inhibitors for cyclin dependent kinases, particularly CDK 1, CDK2, CDK 4 and CDK 5, more particularly ATP:Proteinphosphotransferase $p34^{cdc2}$ (CDK1), which exhibit a high selectivity as well as high efficiency compared to the inhibitors known in the art.

The solution to the above technical problem is achieved by the embodiments characterized in the claims.

In particular, the present invention relates to the use of indigoid bisindole derivatives for the manufacture of a medicament for inhibiting cyclin dependent kinases, particularly CDK 1, CDK2, CDK 4 and CDK 5, more particularly ATP:Proteinphosphotransferase $p34^{cdc2}$ (CDK1), in mammals, preferably in man. Preferably, the indigoid bisindole derivatives are selected from indigo derivatives, isoindigo derivatives or indirubine derivatives.

In a preferred embodiment of the present invention, the indirubine derivate is a compound having the general formula (I)



(I)

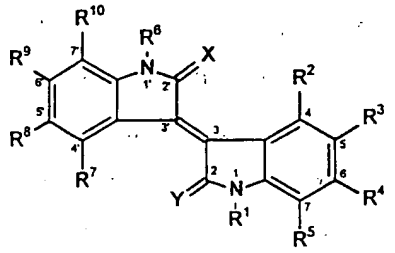
wherein the groups R^1 and R^6 can be the same or different and represent a hydrogen atom; a halogen atom; a hydroxy group; a methylenehydroxy group; a straight-chain or branched-chain alkyl group having 1 to 18 carbon atoms; a straight-chain or branched-chain alkyloxy group having 1 to 18 carbon atoms; a straight-chain or branched-chain methylenealkoxy group having 1 to 18 carbon atoms; a cycloalkyl group having 3 to 7 carbon atoms which can comprise one or more heteroatoms; a substituted or unsubstituted aryl group which can comprise one or more heteroatoms; a substituted or unsubstituted aralkyl group which can comprise one or more heteroatoms; a substituted or unsubstituted aryloxy group which can comprise one or more heteroatoms; a mono-, di- or trialkylsilyl group having 1 to 6 carbon atoms independently of each other in each instance in the straight-chain or branched-chain alkyl group; a mono-, di- or triarylsilyl group with substituted or unsubstituted aryl groups independently of each other in each instance; a trifluoromethyl group; a -COM group; a -COOM group; a $-CH_2COOM$ group, wherein M is hydrogen, a straight-chain or branched-chain alkyl group having 1 to 18 carbon atoms which can additionally carry one or more hydroxy and/or amino groups, or an aryl group which can comprise one or more heteroatoms and can be substituted with one or more halogen atoms, one or more alkyl groups or one or more alkoxy groups; a $-NR^{11}R^{12}$ group, wherein R^{11} and R^{12} can be the same or different and represent a hydrogen atom, a straight-chain or branched-chain alkyl group having 1 to 18 carbon atoms which can additionally carry one or more hydroxy and/or amino groups, a substituted or unsubstituted aryl group which can

comprise one or more heteroatoms; or an acyl group; a methylene-amino group $-CH_2-NR^{11}R^{12}$, wherein R^{11} and R^{12} have the above definitions; a benzyl group, wherein the benzene nucleus can comprise one or more heteroatoms; a methylenecycloalkyl group having 3 to 7 carbon atoms which can comprise one or more heteroatoms; a physiological amino acid residue bound to the nitrogen as an amide; an O-glycoside or a N-glycoside, wherein the glycoside is selected from monosaccharides or disaccharides; or a methylenesulfonate group; R^2, R^3, R^4, R^5, R^7, R^8, R^9 and R^{10} can be the same or different and represent a hydrogen atom; a halogen atom; a hydroxy group; a nitroso group; a nitro group; an alkoxy group; a straight-chain or branched-chain alkyl group having 1 to 18 carbon atoms which can additionally carry one or more hydroxy and/or amino groups; a substituted or unsubstituted aryl group which can comprise one or more heteroatoms; a substituted or unsubstituted aralkyl group which can comprise one or more heteroatoms; a substituted or unsubstituted aryloxy group which can comprise one or more heteroatoms; a substituted or unsubstituted methylenearyloxy group which can comprise one or more heteroatoms; a cycloalkyl group having 3 to 7 carbon atoms which can comprise one or more heteroatoms; a methylenecycloalkyl group having 3 to 7 carbon atoms which can comprise one or more heteroatoms; a trifluoromethyl group; a -COM group; a -COOM group; a $-CH_2COOM$ group, wherein M is hydrogen, a straight-chain or branched-chain alkyl group having 1 to 18 carbon atoms which can additionally carry one or more hydroxy and/or amino groups, or an aryl group which can comprise one or more heteroatoms and can be substituted with one or more halogen atoms, one or more alkyl groups or one or more alkoxy groups; a $-NR^{11}R^{12}$ group, wherein R^{11} and R^{12} can be the same or different and represent a hydrogen atom, a straight-chain or branched-chain alkyl group having 1 to 18 carbon atoms which can additionally carry one or more hydroxy and/or amino groups, a substituted or unsubstituted aryl group which can comprise one or more heteroatoms, or an acyl group, or wherein the nitrogen atom is part of a cycloalkyl group having 3 to 7 carbon atoms which can comprise one or more heteroatom(s); a $-CONR^{11}R^{12}$ group, wherein R^{11} and R^{12} have the above definitions; a hydroxylamino group; a phosphate group; a phosphonate group; a sulfate group; a sulfonate group; a sulfonamide group; a

$-SO_2NR^{11}R^{12}$ group, wherein R^{11} and R^{12} have the above definitions; an azo group $-N=N-R^{13}$, in which R^{13} represents an aromatic system which can be substituted by one or more carboxyl groups, phosphoryl groups or sulfonate groups; or a O-glycoside or a N-glycoside, wherein the glycoside is selected from monosaccharides or disaccharides; or R^1 and R^5, and R^6 and R^{10}, respectively, form independently from each other a ring together having 1 to 4, optionally substituted, CH_2 groups; and X and Y can be the same or different and represent an oxygen atom; a sulfur atom; a selenium atom; a tellurium atom; a NR^{14} group in which the group R^{14} represents a hydrogen atom, a straight-chain or branched-chain alkyl group having 1 to 18 carbon atoms which can be substituted by one or more carboxyl groups, phosphoryl groups or sulfonate groups, a substituted or unsubstituted aryl group which can comprise one or more heteroatoms, an aralkyl group, or a sulfonate group; or a NOR^{14} group, wherein the group R^{14} has the above definitions.

With respect to the benzene nuclei constituting the indirubine derivates of the the above general formula (I) one or more ring atoms can be replaced by nitrogen atoms. Further, one or more aromatic or non-aromatic ring systems which can comprise one or more heteroatoms independently of each other, can be condensed to the indirubine system. Furthermore, the indirubine derivatives having the above general formula (I) can also be bound to a polyethyleneglycolester or a polyethyleneglycolether by ester bondings or ether bondings, respectively.

In another embodiment of the present invention, the isoindigo derivate is a compound having the general formula (II)

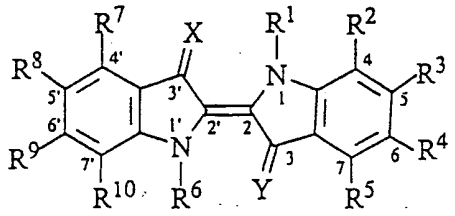


(II)

wherein R^1 to R^{14} and X and Y have the above definitions.

With respect to the benzene nuclei constituting the isoindigo derivates of the above general formula (II) one or more ring atoms can be replaced by nitrogen atoms. Further, one or more aromatic or non-aromatic ring systems which can comprise one or more heteroatoms independently of each other, can be condensed to the isoindigo system. Furthermore, the isoindigo derivatives having the above general formula (II) can be bound to a polyethyleneglycolester or a polyethyleneglycolether by ester bondings or ether bondings, respectively.

In a further embodiment of the present invention, the indigo derivate is a compound having the general formula (III)



(III)

wherein R^1 to R^{14} and X and Y have the above definitions.

With respect to the benzene nuclei constituting the indigo derivates of the above general formula (III) one or more ring atoms can be replaced by nitrogen atoms. Further, one or more aromatic or non-aromatic ring systems which can comprise one or more heteroatoms independently of each other, can be condensed to the indigo system. Furthermore, the indigo derivatives having the above general formula (III) can be bound to a polyethyleneglycolester or a polyethyleneglycolether by ester bondings or ether bondings, respectively.

The above indigoid bisindole derivatives having the general formulas (I), (II) or

(III) can also be in the form of their physiologically acceptable salts.

In the search for new selective inhibitors of cellular signalling pathways, it has surprisingly been found that indigoid bisindole derivatives are highly selective inhibitors of the enzyme complex $p34^{cdc2}$/cyclinB. An inhibition of $p34^{cdc2}$-kinase by indigoid bisindole derivatives is not described in the prior art. Surprisingly, it has turned out that indigoid bisindole derivatives are both highly selective and highly effective inhibitors of cdc2-kinase and other cyclin dependent kinases (CDK's), showing IC_{50}-values with the isolated enzyme down to submicromolar range. The inhibition of CDK-activities also is observed in cell culture, using human tumor cell lines. As an example, indirubin-3'-monoxime was found to inhibit histone H1 phosphorylation as a measure for CDK1/cyclin B activity after 24 h incubation of MCF-7 mammary carcinoma cells. Moreover, the content of cyclin B complex was significantly reduced. Cells arrested by serum deprivation after treatment for 24 h with indirubin-3'-monoxime in serum containing medium exhibited an arrest in G1-phase of the cell cycle at low micromolar concentrations of the substance. In concentrations $\geq$ 5 μM, an additional arrest at G2/M-phase of the cell cycle became apparent. Cells arrested in G2/m by Nocodazole treatment after release of the block exhibited a significant increase of G2/M arrested cells under treatment with $\geq$ 5 μM indirubin-3'-monoxime, resulting in a massive accumulation of cells in G2/M. Concomitantly with the observed intracellular effects, growth inhibition was induced in the same concentration range, resulting in an IC_{50}-value of 3.3 $\pm$0.7 μM after 3 days incubation (IC_{50} = concentration that induces 50% growth inhibition as compared to vehicle control). In addition, induction of apoptotic cell death was observed.

Based on the above mentioned selective inhibitory potency down to the nanomolar range, indirubine derivatives can be used for analytical biochemistry, especially for the study of cell cycle effects. Furthermore, these compounds can be used for the treatment of diseases in patients, which are connected to the loss of proliferation control without any restriction to these potential areas of application. These include cancers, psoriasis, cardiovascular diseases

(stenosis, restenosis) (Brooks et al., J. Biol. Chem., 272, (1997), 29207-2921-1), infectious diseases (unicellular parasites (Trypanosoma, Toxoplasma, Plasmodium, etc.), fungi, etc.), nephrology (glomerulonephritis: Pippin et al., J. Clin. Invest., 100, (1997), 2512-2520), neurodegenerative disorders such as Alzheimer disease (Imahori, K., Uchida T., J. Biochem., 121, (1997), 179-188), viral infections such as cytomegalovirus (Bresnahan W. A. et al., Virology 231,(1997), 239-247) and HIV (Mancebo H.S.Y. et al., Genes & Dev., 11, (1997), 2633-2644).

The present invention is explained further by the following examples:

Example 1: Synthesis of indirubine

To a solution of 0.42 g (2.4 mmol) of indoxyl acetate in 20 ml methanol under argon 0.35 g (2.4 mmol) of isatin and 0.55 g (5.2 mmol) of sodium carbonate are added. The mixture is stirred for 30 min at ambient temperature. After 24 h standing at ambient temperature, the reaction mixture is filtered off. The precipitate is washed with little methanol and water until the filtrate shows a neutral pH. Residual water is removed by storage in an evacuated exsiccator over potassium hydroxide. Recrystallisation from ethanol or pyridine gives deep purple crystals (Russell G.A., Kaupp G. (1969), J. Am. Chem. Soc., 91, 3851-9, modified).

Yield: 0.51 g (81%), fine, deep-purple needles, Fp: 341-343°C

CHN-analysis: ($C_{16}H_{10}N_2O_2$); MW: 262,26 g/mol; calc.: 73.3% C, 3.8% H, 10.7% N; found: 73.2% C, 4.0% H, 10.6% N

mass spectrum: m/z = 262: (M^+, 100%), 234: (43%), 205 (25%), 158 (3%), 131 (4%), 103 (7%), 76 (3%)

^{1}H-NMR and ^{13}C-NMR-spectrum are in accordance with the proposed structure.

IR-spectrum: 3340 cm^{-1}: ν (N-H), 1710 cm^{-1}: ν (3´-C=O), 1650 cm^{-1}: ν (2-C=O), 1590 cm^{-1}: ν (C=C, aryl), 1450 cm^{-1}: ν (C=C, aryl), 745 cm^{-1}: ν (aryl with four neighbouring H-atoms).

UV/Vis-spectrum (DMSO): 290 nm, 363 nm, 383 nm (shoulder), 551nm

Essentially the same synthetic procedere was applied for the following Examples 2 to 9 and 11 and 12:

Example 2: 5-Iodoindirubine

Yield: 80%, fine, deep-purple needles, Fp: 334-335°C (decomposition);
CHN-analysis ($C_{16}H_9IN_2O_2$); MG = 388.16 g/mol; calc.: 49.5% C, 2.3% H, 7.2% N; found.: 49.7% C, 2.5% H, 7.1% N;
Mass spectrum: 388 (M^+, 100%), 360 (3%), 269 (9%), 261 (6%), 233 (16%), 205 (16%), 128 (1%);
^{1}H-NMR- and ^{13}C-NMR-spectrum are in accordance with the proposed structure.
UV/Vis-spectrum (DMSO): 370 nm, 386 nm (shoulder), 555 nm.

Example 3: 5-Bromoindirubine

Yield: 70%, fine, deep-purple needles;
CHN-analysis ($C_{16}H_9BrN_2O_2$); MG = 341.16 g/mol, calc.: 56.3% C, 2.7% H, 8.2% N; found 56.4% C, 2.7% H, 8.2% N;
Mass spectrum: 342(M^+, 100%), 340 (M^+, 99%), 314 (18%), 262 (64%), 233 (34%), 205 (81%), 177 (10%);
^{1}H-NMR- and ^{13}C-NMR-spectrum are in accordance with the proposed structure.

Example 4: 5-Chloroindirubine

Yield: 95%, fine, deep-purple needles;
CHN-analysis ($C_{16}H_9ClN_2O_2$); MG = 296.70 g/mol; calc.: 49.5% C, 2.3% H, 7.2% N; found: 49.7% C, 2.5% H, 7.1% N;
Mass spectrum: m/z = 296 (M^+, 100%), 268 (39%), 239 (8%), 233 (35%), 205 (50%), 177 (7%), 153 (6%), 137 (7%), 77 (7%), 120 (4%), 102 (6%), 77 (7%).
^{1}H-NMR- and ^{13}C-NMR-spectrum are in accordance with the proposed structure.

Example 5: 5-Fluoroindirubine

Yield: 92%, fine, deep-purple needles;
CHN-analysis ($C_{16}H_9FN_2O_2$), MG = 280.25 g/mol, calc.: 68.6% C, 3.2% H, 9.9% N; found: 68.0%C, 3.2% H, 9.9% N;

Mass spectrum: m/z = 281 ($M^+ + H^+$, 19%), 280 (M^+, 100%), 252 (73%), 223 (32%), 176 (6%), 140 (7%), 121 (13%), 94 (4%), 76 (12%), 77 (7%), 57 (4%), 44(15%).

^{1}H-NMR- and ^{13}C-NMR-spectrum are in accordance with the proposed structure.

Example 6: 5-Methylindirubine:

Yield: 92%, fine, deep-purple needles;

CHN-analysis ($C_{17}H_{12}N_2O_2$), MG = 276.28 g/mol, calc.: 73.9% C, 4.4% H, 10.1% N; found: 73.8%C, 4.3% H, 10.2% N;

Mass spectrum: m/z = 276 (M^+, 100%), 261 (10%), 248 (47%), 247 (53%), 220 (6%), 219 (18%), 205 (7%), 171 (4%), 165 (10%), 138 (4%), 133 (15%), 104 (7%), 77 (7%);

^{1}H-NMR- and ^{13}C-NMR-spectrum are in accordance with the proposed structure.

Example 7: 5-Nitroindirubine

Yield: 88%, fine, deep-purple needles;

CHN-analysis ($C_{16}H_9N_3O_4$), MG = 307.26 g/mol; calc.: 62.5% C, 3.0% H, 13.7% N; found: 62.4%C, 3.0% H, 13.3% N;

Mass spectrum: m/z = 307 (M^+, 5%), 276 (10%), 262 (100%), 234 (23%), 205 (22%), 158 (6%), 131 (10), 104 (19%), 76 (12%), 50 (6%).

^{1}H-NMR- and ^{13}C-NMR-spectrum are in accordance with the proposed structure.

Example 8: 5'-Bromoindirubine

Yield: 92%, fine, deep-purple needles;

CHN-analysis ($C_{16}H_9BrN_2O_2$), MG = 341.16 g/mol, calc.: 56.3% C, 2.7% H, 8.2% N; found: 55.7%C, 2.5% H, 8.0% N.

^{1}H-NMR- and ^{13}C-NMR-spectrum are in accordance with the proposed structure.

Example 9: 5,5'-Dibromoindirubine

Yield: 94%, fine, deep-purple needles;

CHN-analysis ($C_{16}H_8Br_2N_2O_2$), MG = 420.06 g/mol; calc.: 45.7% C, 1.9% H, 6.7% N; found: 45.8% C, 2.0% H, 6.4% N.

^{1}H-NMR-spectrum is in accordance with the proposed structure.

Example 10: Indirubine-3'-oxime

Indirubine-3'-oxime was synthesized by reaction of indirubine with hydroxylamine hydrochloride in a pyridine solution (Farbwerke vorm. Meister Lucius & Brüning in Hoechst a.M., Patentschrift des Reichspatentamtes Nr. 283726 (1913)). ^{13}C-NMR-spectroscopy revealed the location of the hydroxyimino residue in 3'-Position (δ(C2) = 171.05 ppm; δ(C3') = 145.42 ppm; DMSO-d_6, RT)

Yield: 90 %, red crystals;

CHN-analysis ($C_{16}H_{11}N_3O_2$), MG = 277.30g/mol; calc:: 69.3% C, 4.0% H, 15.2 % N; found: 69.0% C, 4.0% H, 14.9% N;

^{1}H-NMR- and ^{13}C-NMR-spectrum are in accordance with the proposed structure.

Example 11: Indirubine-5-sulfonic acid

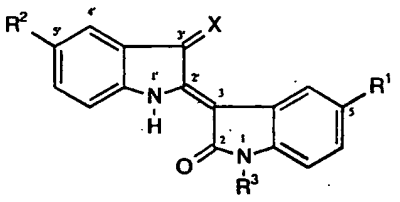
Yield: 76%, crystalline, deep-purple substance;

Mass spectrum: 388 (M^+, 100%), 360 (3%), 269 (9%), 261 (6%), 233 (16%), 205 (16%), 128 (1%).

^{1}H-NMR- and ^{13}C-NMR-spectrum are in accordance with the proposed structure.

Table 2 shows the structures of the compunds of Examples 1 to 11.

Tab. 2:



Example	compound	R^1	R^2	R^3	X
1	Indirubine	H	H	H	O
2	5-Iodoindirubine	I	H	H	O
3	5-Bromoindirubine	Br	H	H	O
4	5-Chloroindirubine	Cl	H	H	O
5	5-Fluoroindirubine	F	H	H	O
6	5-Methylindirubine	CH_3	H	H	O
7	5-Nitroindirubine	NO_2	H	H	O
8	5'-Bromoindirubine	H	Br	H	O
9	5,5'-Dibromoindirubine	Br	Br	H	O
10	Indirubine-3'-oxime	H	H	H	NOH
11	Indirubine-5-sulfonic acid	SO_3H	H	H	O

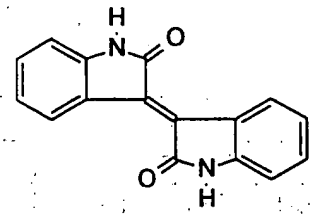
Table 3 shows the specificity of the compounds of Examples 1 to 11 (IC_{50}-values given in μM) to inhibit cdc2 kinase in comparison to other cellular kinases.

Tab. 3:

Example	cdc2	cdc25	PKA	PKC
1	2	150	no Effect	no Effect
2	0.3	130	no Effect	no Effect
3	0.35	32	ND	ND
4	0.40	55	ND	ND
5	2.0	70	ND	ND
6	0.8	60	ND	ND
7	2.2	60	ND	ND
8	6.5	ND	ND	ND
9	7	ND	ND	ND
10	0.18	ND	ND	ND
11	0.055	110	ND	ND

ND: not dedected; PKA: cAMP-dependent protein kinase;
PKC: Ca^{2+}-dependent protein kinase

Example 12: Isoindigo



Isoindigo was synthesized by reaction of oxindole with isatin in acetic acid with addition af hydrochloric acid (Wahl A., Bayard P., Comptes Rendues Hebdomadaires des Seances de L'Academie des Sciences, 148, (1909), 716-719).

Yield: 84%, crystalline, brown substance;

CHN-analysis ($C_{16}H_{10}N_2O_2$), MG = 262.26 g/mol; calc.: 73.3% C, 3.8% H, 10,7% N; found: 73.0% C, 3.8% H, 10.9% N;

Mass spectrum: m/z = 262 (M^+, 100%), 234 (85%), 220 (5%), 205 (18%), 190 (4%), 177 (5%), 151 (5%), 132 (17%), 103 (6%), 76 (4%), 32 (26%).

^{1}H-NMR- and ^{13}C-NMR-spectrum are in accordance with the proposed structure.

Isoindigo shows an IC_{50}-value of 80μM for the $p34^{cdc2}$/cyclinB complex.

Table 4 shows the kinase inhibition selectivity of indigo, indirubin, 5-chloroindirubine, indirubine-3'-monoxim and indirubine-5 sulfonic acid. The indicated IC_{50} values were calculated from respective dose response curves and are presented in μM:

Tab. 4:

Enzyme	indigo	indirubine	5-chloro-indirubine	indirubine-3'-monoxime	indirubine-5-sulfonic acid
CDK1/ cyclin B	>1000.000	10.000	0.400	0.180	0.055
CDK2/ cyclin A	70.000	2.200	0.750	0.440	0.035
CDK2/ cyclin E	> 1000.000	7.500	0.550	0.250	0.150
CDK4/ cyclin D1	> 100.000	12.000	6.500	n.t.	0.300
CDK5/ p35	> 100.000	5.500	0.800	0.100	0.065

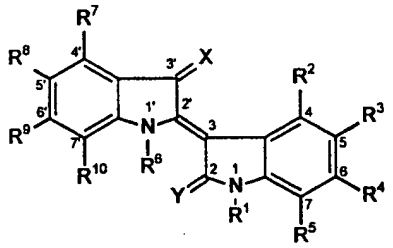
Claims

1. Use of indigoid bisindole derivatives for the manufacture of a medicament for inhibiting cyclin dependent kinases.

2. Use according to claim 1, wherein the cyclin dependent kinases are selected from CDK 1, CDK 2, CDK 4 or CDK5.

3. Use according to claim 1 or 2, wherein the indogoid bisindole derivatives are selected from indigo derivatives, isoindigo derivatives or indirubine derivatives.

4. Use according to claim 3, wherein the indirubine derivate is a compound having the general formula (I)



(I)

wherein the groups R^1 and R^6 can be the same or different and represent a hydrogen atom; a halogen atom; a hydroxy group; a methylenehydroxy group; a straight-chain or branched-chain alkyl group having 1 to 18 carbon atoms; a straight-chain or branched-chain alkyloxy group having 1 to 18 carbon atoms; a straight-chain or branched-chain methylenealkoxy group having 1 to 18 carbon atoms; a cycloalkyl group having 3 to 7 carbon atoms which can comprise one or more heteroatoms; a substituted

or unsubstituted aryl group which can comprise one or more heteroatoms; a substituted or unsubstituted aralkyl group which can comprise one or more heteroatoms; a substituted or unsubstituted aryloxy group which can comprise one or more heteroatoms; a mono-, di- or trialkylsilyl group having 1 to 6 carbon atoms independently of each other in each instance in the straight-chain or branched-chain alkyl group; a mono-, di- or triarylsilyl group with substituted or unsubstituted aryl groups independently of each other in each instance; a trifluoromethyl group; a -COM group; a -COOM group; a $-CH_2COOM$ group, wherein M is hydrogen, a straight-chain or branched-chain alkyl group having 1 to 18 carbon atoms which can additionally carry one or more hydroxy and/or amino groups, or an aryl group which can comprise one or more heteroatoms and can be substituted with one or more halogen atoms, one or more alkyl groups or one or more alkoxy groups; a $-NR^{11}R^{12}$ group, wherein R^{11} and R^{12} can be the same or different and represent a hydrogen atom, a straight-chain or branched-chain alkyl group having 1 to 18 carbon atoms which can additionally carry one or more hydroxy and/or amino groups, a substituted or unsubstituted aryl group which can comprise one or more heteroatoms; or an acyl group; a methyleneamino group $-CH_2-NR^{11}R^{12}$, wherein R^{11} and R^{12} have the above definitions; a benzyl group, wherein the benzene nucleus can comprise one or more heteroatoms; a methylenecycloalkyl group having 3 to 7 carbon atoms which can comprise one or more heteroatoms; a physiological amino acid residue bound to the nitrogen as an amide; an O-glycoside or a N-glycoside, wherein the glycoside is selected from monosaccharides or disaccharides; or a methylenesulfonate group; R^2, R^3, R^4, R^5, R^7, R^8, R^9 and R^{10} can be the same or different and represent a hydrogen atom; a halogen atom; a hydroxy group; a nitroso group; a nitro group; an alkoxy group; a straight-chain or branched-chain alkyl group having 1 to 18 carbon atoms which can additionally carry one or more hydroxy and/or amino groups; a substituted or unsubstituted aryl group which can comprise one or more heteroatoms; a substituted or unsubstituted aralkyl group which can comprise one or more heteroatoms; a substituted or unsubstituted aryloxy

group which can comprise one or more heteroatoms; a substituted or unsubstituted methylenearyloxy group which can comprise one or more heteroatoms; a cycloalkyl group having 3 to 7 carbon atoms which can comprise one or more heteroatoms; a methylenecycloalkyl group having 3 to 7 carbon atoms which can comprise one or more heteroatoms; a trifluoromethyl group; a -COM group; a -COOM group; a $-CH_2COOM$ group, wherein M is hydrogen, a straight-chain or branched-chain alkyl group having 1 to 18 carbon atoms which can additionally carry one or more hydroxy and/or amino groups, or an aryl group which can comprise one or more heteroatoms and can be substituted with one or more halogen atoms, one or more alkyl groups or one or more alkoxy groups; a $-NR^{11}R^{12}$ group, wherein R^{11} and R^{12} can be the same or different and represent a hydrogen atom, a straight-chain or branched-chain alkyl group having 1 to 18 carbon atoms which can additionally carry one or more hydroxy and/or amino groups, a substituted or unsubstituted aryl group which can comprise one or more heteroatoms, or an acyl group, or wherein the nitrogen atom is part of a cycloalkyl group having 3 to 7 carbon atoms which can comprise one or more heteroatom(s); a $-CONR^{11}R^{12}$ group, wherein R^{11} and R^{12} have the above definitions; a hydroxylamino group; a phosphate group; a phosphonate group; a sulfate group; a sulfonate group; a sulfonamide group; a $-SO_2NR^{11}R^{12}$ group, wherein R^{11} and R^{12} have the above definitions; an azo group $-N=N-R^{13}$, in which R^{13} represents an aromatic system which can be substituted by one or more carboxyl groups, phosphoryl groups or sulfonate groups; or a O-glycoside or a N-glycoside, wherein the glycoside is selected from monosaccharides or disaccharides; or R^1 and R^5, and R^6 and R^{10}, respectively, form independently from each other a ring together having 1 to 4, optionally substituted, CH_2 groups; and X and Y can be the same or different and represent an oxygen atom; a sulfur atom; a selenium atom; a tellurium atom; a NR^{14} group in which the group R^{14} represents a hydrogen atom, a straight-chain or branched-chain alkyl group having 1 to 18 carbon atoms which can be substituted by one or more carboxyl groups, phosphoryl groups or sulfonate groups, a substituted or unsubstituted aryl group which can comprise one or more heteroatoms, an

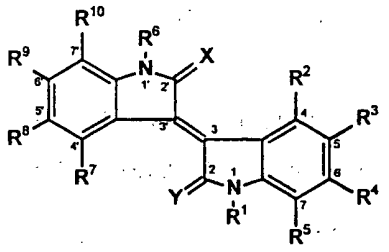
aralkyl group, or a sulfonate group; or a NOR^{14} group, wherein the group R^{14} has the above definitions.

5. Use according to claim 4, wherein one or more ring atoms of the benzene nuclei of the compound having the general formula (I) are replaced by nitrogen atoms.

6. Use according to claim 4 or 5, wherein one or more aromatic or non-aromatic ring systems which can comprise one or more heteroatoms independently of each other, are condensed to the indirubine system.

7. Use according to anyone of claims 4 to 6, wherein the compound having the general formula (I) is bound to a polyethyleneglycolester or a polyethyleneglycolether.

8. Use according to claim 3, wherein the isoindigo derivate is a compound having the general formula (II)



(II)

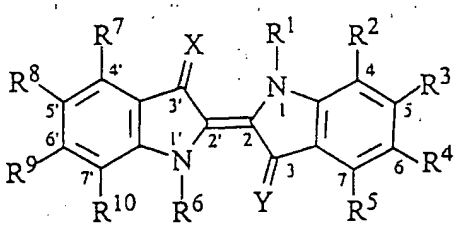
wherein R^1 to R^{14} and X and Y have the meanings as defined in claim 4.

9. Use according to claim 8, wherein one or more ring atoms of the benzene nuclei of the compound having the general formula (II) are replaced by nitrogen atoms.

10. Use according to claim 8 or 9, wherein one or more aromatic or non-aromatic ring systems which can comprise one or more heteroatoms independently of each other, are condensed to the isoindigo system.

11. Use according to anyone of claims 8 to 10, wherein the compound having the general formula (II) is bound to a polyethyleneglycolester or a polyethyleneglycolether.

12. Use according to claim 3, wherein the indigo derivate is a compound having the general formula (III)



(III)

wherein R^1 to R^{14} and X and Y have the meanings as defined in claim 4.

13. Use according to claim 12, wherein one or more ring atoms of the benzene nuclei of the compound having the general formula (III) are replaced by nitrogen atoms.

14. Use according to claim 12 or 13, wherein one or more aromatic or non-aromatic ring systems which can comprise one or more heteroatoms independently of each other, are condensed to the indigo system.

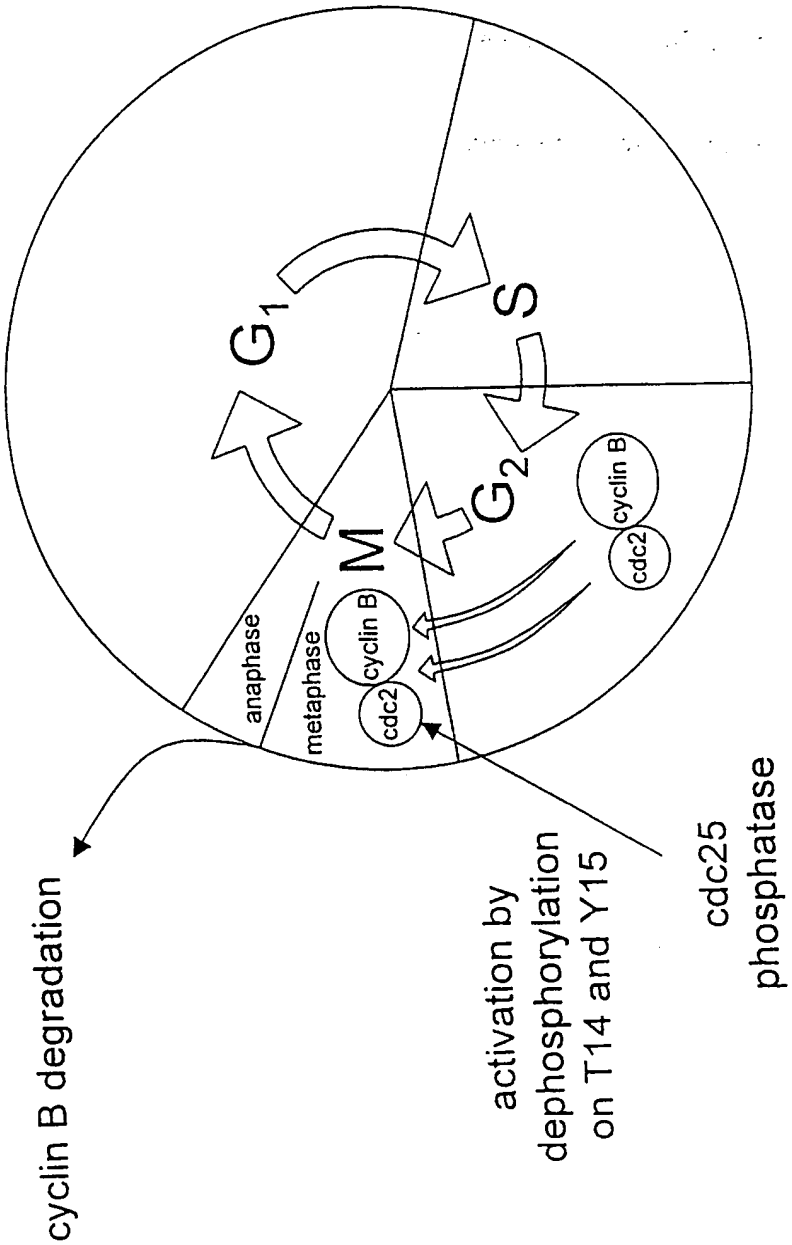
15. Use according to anyone of claims 12 to 14, wherein the compound having the general formula (III) is bound to a polyethyleneglycolester or a polyethyleneglycolether.

16. Use according to anyone of claims 1 to 15, wherein the indigoid bisindole derivative is in the form of a physiologically acceptable salt.

17. Compound which is 5-fluoroindirubine.

18. Compound which is 5-nitroindirubine.

19. Compound which is 5,5'-dibromoindirubine.



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(54) Title: USE OF INDIGOID BISINDOLE DERIVATIVES FOR THE MANUFACTURE OF A MEDICAMENT TO INHIBIT CYCLIN DEPENDENT KINASES

(57) Abstract

The present invention relates to the use of indigoid bisindole derivatives for the manufacture of a medicament for inhibiting cyclin dependent kinases, particularly CDK 1, CDK 2, CDK 4 and CDK 5, more particularly ATP:Proteinphosphotransferase $p34^{cdc2}$ (CDK1).

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 99/03625

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/40 C07D209/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	HOESSEL, RALPH ET AL: "Indirubin, the active constituent of a Chinese antileukemia medicine, inhibits cyclin-dependent kinases" NAT. CELL BIOL. (1999), 1(1), 60-67 , XP002122993 the whole document --- -/--	1-4,8, 12,16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
24 November 1999	08/12/1999

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Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US JI, XIUJUAN ET AL: "Antineoplastic effect of indirubin derivatives and their structure-activity relations" retrieved from STN Database accession no. 103:98313 XP002123718 abstract & YAOXUE XUEBAO (1985), 20(2), 137-9 , ---	1-4,8, 12,16
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Y	DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US GU, Y. C. ET AL: "Synthesis of some halogenated indirubin derivatives" retrieved from STN Database accession no. 112:178548 XP002123720 abstract & YAOXUE XUEBAO (1989), 24(8), 629-32 , ---	1-4,8, 12,16
Y	PATENT ABSTRACTS OF JAPAN vol. 010, no. 148 (C-350), 29 May 1986 (1986-05-29) & JP 61 007254 A (ISUKURA SANGYO KK), 13 January 1986 (1986-01-13) abstract ---	1-4,8, 12,16
Y	WO 98 07695 A (HIRTH KLAUS PETER ;SHAWVER LAURA KAY (US); SUGEN INC (US); TANG PE) 26 February 1998 (1998-02-26) claims ---	1-4,8, 12,16
A	HUNTER T ET AL: "CYCLINS AND CANCER II: CYCLIN D AND CDK INHIBITORS COME OF AGE" CELL,US,CELL PRESS, CAMBRIDGE, NA, vol. 79, page 573-582 XP002069793 ISSN: 0092-8674 the whole document --- -/--	1-4,8, 12,16

INTERNATIONAL SEARCH REPORT

Inter ional Application No
PCT/EP 99/03625

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	ARTUR BURGER ET AL.: "Pharmaceutisches Wörtebuch" 1998 , DE GRUITER , BERLIN XP002123717 page 704-705	1,2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 99/03625

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 5,6,9,10,13,14 and part of 7,11,15,16

Present claims 1-2 relate to the manufacture of a medicament for inhibiting cyclin dependent kinases (CDK). This, however, is not a specified therapeutic application. Implicitly this medicament is intended for the treatment of diseases related to CDK modulation; the diseases for which the medicament is intended are however not defined in the claims. The use of the definition "medicament for inhibiting cyclin dependent kinases" is considered to lead to a lack of clarity within the meaning of article 6 PCT. The lack of clarity is such to render a meaningful complete search impossible. Consequently the search has been restricted to the diseases mentioned in the description at pages 9-10.
Present claims 5,6,9,10,13,14, relate to an extremely large number of possible compounds. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for none of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.
Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds
mentioned in the description, in claims 17-19, compounds corresponding to Markush formula I, II and III, closely related compounds, and for the general idea underlying the application.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 99/03625

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 61007254 A	13-01-1986	NONE	
WO 9807695 A	26-02-1998	AU 4155697 A	06-03-1998
		EP 0929520 A	21-07-1999